Master's Thesis of Agriculture

Combined effect of high pressure and vinegar addition on the control of *Clostridium perfringens* and quality in emulsion-type sausage

초고압과 식초 병용처리가 소시지에 접종된
Clostridium perfringens 제어와
품질에 미치는 영향

August, 2018

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Abstract

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The objective of present study was to evaluate the combined effect of high pressure (HP) and vinegar addition on the control of *Clostridium perfringens* and quality in emulsion-type sausage. Emulsion-type sausages were manufactured with different levels of vinegar (0, 1, and 2%) and sodium nitrite (0.02%, positive control), respectively. Sausage samples for microbial analysis were inoculated with vegetative cells and spores of *C. perfringens*, respectively. Then, the sausages were treated with 4 cycles of HP for 3 min at each 500 MPa

except for sodium nitrite group. Samples for microbial analysis were stored for 2 weeks at 4°C and then increased to 20°C and maintained the temperature for 3 weeks, while the other samples for quality analysis were stored for 5 weeks at 4°C. The HP treatment significantly reduced the initial counts of *C. perfringens* vegetative cells in emulsion-type sausage at 4°C. Sausage added with vinegar showed no significant difference with sodium nitrite in the populations of *C. perfringens* vegetative cells and spores, even under abusive temperature at 20°C. The sausages without vinegar showed higher counts of *C. perfringens* spores compared to sodium nitrite, regardless of HP treatment. The HP and vinegar treatment in sausage samples showed higher pH and water holding capacity than sodium nitrite group. The vinegar addition inhibited the lipid oxidation of sausage during the entire storage period, whereas HP treatment increased the lipid oxidation value of the sample. However, vinegar treatment in sausage tended to be tarnished in the color and high in hardness and chewiness value. In conclusion, the combination of HP and vinegar addition would control *C. perfringens* during storage with acceptable quality properties in emulsion-type sausage.

Keywords: *Clostridium perfringens*, Emulsion-type sausage, High pressure, Sodium nitrite, Vinegar

Contents

List of Tables

Chapter I.

Chapter II.

List of Figures

Chapter I.

Chapter II.

List of Abbreviations

AOAC	:	Association of official analytical chemists
BPW	:	Buffered peptone water
CDC	:	Centers for Disease Control and Prevention
CMM	:	Cooked meat medium
CFU	:	Colony-forming units
C. perfringens	:	*Clostridium perfringens*
CPE	:	*Clostridium perfringens* enterotoxin
DPA	:	Dipicolinic acid
DW	:	Distilled water
FSIS	:	Food Safety and Inspection Service
FTG	:	Fluid thioglycollate
GRAS	:	Generally recognized as safe
HP	:	High pressure
HPTP	:	High pressure thermal processing
IARC	:	International Agency for Research on Cancer
KFDA	:	Korea Food and Drug Administration
LLDPE	:	Linear low density polyethylene
MDA	:	Malondialdehyde
MPa	:	Mega-Pascal

PATS	:	Pressure assisted thermal sterilization
PBS	:	Phosphate buffer saline
PC	:	Positive control
PEF	:	Pulsed electric field
RTE	:	Ready-to-eat
SEM	:	Standard error of the mean
TBARS	:	2-Thiobarbituric acid reactive substances
TSC	:	Tryptose-sulfite-cycloserine
USDA	:	United States Department of Agriculture
UV	:	Ultraviolet
WHC	:	Water holding capacity
WHO	:	World Health Organization

Chapter I.

General Introduction

Clostridium perfringens is a gram positive, anaerobic spore-forming bacterium that produces many different toxins (Shimizu et al., 2002). This organism is commonly found in the environment such as soil, dust, water and food as well as gastrointestinal tract of humans and animals (Juneja et al., 2009). The *C. perfringens* strains are classified as A, B, C, D, or E depending on isotype (Uzal & McClane, 2011). The *C. perfringens* type A food poisoning ranks as the third most common foodborne disease after norovirus and *Salmonella* spp. (Havelaar et al., 2012). Illness is caused after ingestion of contaminated food that subsequently produces toxin, causing a spectrum of symptoms (Evelyn & Silva, 2016). These symptoms involve vomiting, abdominal pain, and stomach cramps, followed by diarrhea.

A majority of the foods implicated in *C. perfringens* foodborne illness were meat products (Olsen et al., 2000), and has often been associated with mass catering including hospitals, hotels, schools, and factories, and even homes. Outbreaks due to *C. perfringens* typically arises following inappropriate temperature control of cooked meat products during cooling and storage which spores germinate then grow rapidly due to their short generation times (Bauer et al., 1981). The United States Department of Agriculture (USDA) Food Safety and Inspection Service (FSIS) compliance

guidelines recommended cooling of cooked meat from 54.4 to 26.7°C within 1.5 h and further to 4.4°C within an additional 5 h (USDA, 2001). Unfortunately, this practices have not been always followed strictly, and therefore the inactivation of *C. perfringens* spores through other processing is an alternative strategy (Evelyn & Silva, 2016). The thermal processing of *C. perfringens* in meat products has been reported in several studies (Byrne et al., 2006; Juneja et al., 2006). However, *C. perfringens* spores are a heat-resistant and can survive 1 h at 100°C (Labbé et al., 2014). Thus, most thermal inactivation studies have been carried out at more than 100°C (Orsburn et al., 2008; Sarker et al., 2000). The thermal processing for a long time at high temperature might cause adverse effects in meat color, texture, juiciness, and off-flavor (Jayasena et al., 2013).

High pressure (HP) is a non-thermal technology which is being used as a final decontamination treatment for vacuum-packed meat products (Slongo et al., 2009). HP is attractive as it can kill vegetative cells of microorganisms and improve safety and shelf life of the products, without significantly affecting on nutritional and quality properties compared to conventional thermal processing (Garriga et al., 2004). HP treatment for 5-10 min at 400-600 Mega-Pascal (MPa) is typically applied to inactivate pathogenic microorganisms and to extend shelf life of food (Evelyn & Silva, 2016). Unfortunately, under normal processing conditions, HP cannot inactivate bacterial endospores and the pressure resistance of *C. perfringens* spores is well reported (Margosch et al., 2006; Reddy et al., 1999). It must be coupled with elevated temperatures and/or required HP level to inactivation spores. Evelyn and Silva (2016) reported that efficacy of high pressure thermal processing using 600 MPa at 82°C for

1.3 min could be achieved 6D reduction of *C. perfringens* spores in beef slurry. D_{10} value was calculated the exposure time required to reduce 90% of microorganism population. Bacterial spores can survive even an applied pressure of 1200 MPa at room temperature (Larson et al., 1918). However, high temperature or pressure level raise the process time and cost in food industry as well as change food quality parameters. Therefore, it must be combined with commercial non-thermal HP and some other alternatively treatment to control *C. perfringens* spores.

Current strategies to control *C. perfringens* germination and outgrowth involve chemical additives. In general, meat processors add sodium nitrite to processed meat products as a color-fixing agent, inhibition of lipid oxidation as well as a major role in the control foodborne pathogens, including *Clostridium* species. Despite all of its beneficial functions, consumer concerns about safety of nitrite to human health. Nitrite can cause the formation of carcinogenic *N*-nitrosamines in the gastrointestinal tract (Butler, 2015). Since, consumer demand for organic and natural meat products has increased due to the concerns of synthetic additives, the meat industry continues to search for alternative additives to produce nitrite-free meat products (Alahakoon et al., 2015).

Organic acids are used as food preservatives and have been found to act as inhibitory agents for *C. perfringens* (Talukdar et al., 2017). Acetic acid, which is mostly used as vinegar, has been successfully applied for microbial inhibition in variety of foods (Ray, & Bhunia, 2008). Vinegar has been used as a food ingredient for the purpose of flavoring and preserving foods for thousands of years (Johnston & Gaas, 2006). Valenzuela-Martinez et al. (2010) reported that buffered vinegar at 3.5%

could inhibit *C. perfringens* spore germination and outgrowth in ground turkey roast. In addition, vinegar additives in meat products are suitable for the definition of clean label (i.e., products that do not contain ingredients that can be classified as chemicals) and provide the antimicrobial effect and safety assurance.

In these days, without safety assurance, a lot of nitrite-free meat products are being marketed to meet the consumer expectations. However, there is few literatures on control of *C. perfringens* on nitrite-free meat products. Therefore, the objective of this study is to evaluate the combined effects of HP and vinegar addition on the control of *C. perfringens* and changes on its quality properties in emulsion-type sausage. Samples for microbial analysis were stored at 4°C for 2 weeks then increased to 20°C and maintained the temperature for 3 weeks, while the other samples for quality analysis were stored at 4°C for 5 weeks. Two different storage temperatures, 4°C and 20°C, were chosen to simulate proper refrigeration conditions (4°C) and temperature abuse (20°C) to represent the typical "poor control" conditions during distribution and storage of the product in the food chain that could favor spore germination and outgrowth. Moreover, the conditions in vacuum-packed sausages are suitable for the growth and toxin production by *C. perfringens*, as it is an oxygen-free environment. Commercial pork sausage had 38.9% prevalence of *C. perfringens* (Bauer et al., 1981).

Chapter II.

Literature Review

2.1. *Clostridium perfringens*

2.1.1. What is *C. perfringens*?

Clostridium perfringens is a gram-positive, anaerobic, endospore forming bacterium that is found in many environmental sources soil, dust, water and food as well as intestines both of humans and animals. *C. perfringens* were the most widespread bacteria and sensitive among all organisms (Alahakoon et al., 2015; Uzal et al., 2014). *C. perfringens* strains are grouped into 5 types (types A, B, C, D, and E), which are based on 17 different toxins to cause a spectrum of diseases in humans and animals (Lindström et al., 2011; Smith, 1979). Around 5% of global *C. perfringens* type A produce an enterotoxin (CPE) genes, which cause gastrointestinal infections and diarrhea in humans (Fisher et al., 2005). In addition to producing CPE, *C. perfringens* food borne isolates have the ability to form spores that are highly resistant.

The endospores formation is complex which allows the developing spore to survive (i.e., decades) under extreme environmental conditions when moisture and nutrients are scarce (Reineke et al., 2013). *C. perfringens* spores come in contact with small molecules termed germinates, they return to being vegetative cells via the

germination process (Paredes-Sabja et al., 2008). The matured spore has a well-structured multilayer morphology after sporulation (Fig. 1.; Setlow, 2011). This multilayer and thickness of the bacterial spore coat is believed to account for the strong resistance such as high temperature and toxic chemicals (Reddy et al., 2003). The resistances of spores are different even among the same species (Wilson et al., 2008). *C. perfringens* can grow at temperatures ranging from 15 to 50°C and pH values between 5.0 to 8.0. *C. perfringens* spores germinate then proliferation rapidly due to their generation times as short as 8 min at optimal growth temperatures between 43 to 45°C (Evelyn & Silva, 2016). These facts suggest the importance of developing an effective strategies that could control the growth of different strains of *C. perfringens* (Talukdar et al., 2017).

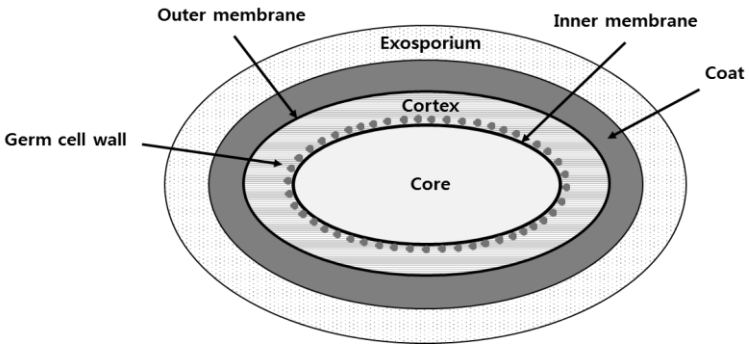

Fig. 1. Schematic structure of a *Bacillus*/*Clostridium* spore. Spore layers are not drawn to scale, the exosporium is not present on spores of some species (adapted from Setlow, 2011)

2.1.2. Outbreaks of *C. perfringens* in animal origin foods

Spore-forming bacteria are special problems for the food industry, particularly the meat industry, and has been implicated in several large outbreaks. According to some estimates, nearly a million illnesses due to *C. perfringens* type A strains occur annually in the United States, resulting in a net economic burden of US $382 million (Uzal et al., 2014). The majority outbreaks of *C. perfringens* illness are associated with consuming contaminated raw meat and meat products such as stews, gravy, casseroles, and cooked sausages. These outbreaks are extremely costly losses in terms of financial and reputation of meat industry. Therefore, *C. perfringens* might become a special problem in meat industry.

Commercial pork sausage had 38.9% prevalence of *C. perfringens* (Bauer et al., 1981). Vacuum-packaged sausages have a longer shelf-life because of the spoilage bacteria destruction after in-package pasteurization process. However, *C. perfringens* were more frequently detected, as a result of decreased competition from the spoilage organisms (Franz & von Holy, 1996). Moreover, vacuum-packaged sausages are suitable for the growth and toxin production by *C. perfringens*, as it is an anaerobic conditions. The most common sources of *C. perfringens* infections are ‘poor control’ that large quantities of meat containing foods stored with insufficient cooling and reheating after cooking (Granum, 1990). Therefore, outbreaks often happen in industrial kitchens, such as hospitals, schools, nursing homes, or even at private homes. Outbreaks of *C. perfringens* were most frequent in restaurants, followed by a catering facility (Grass et al., 2013). That’s why *C. perfringens* referred to as ‘food

service germ' or 'cafeteria germ'. The symptom caused by *C. perfringens* is vomiting, abdominal cramps, and diarrhea which usually occurs 6 to 24 hour after ingestion of a food containing large number of cells that produce toxin. Although there has been significantly studies in inactivating *C. perfringens* vegetative cells through various technologies, the inactivation of spores from meat products still remains a challenge (Talukdar et al., 2017).

2.1.3. Control of *C. perfringens*

2.1.3.1. Traditional methods

Traditional thermal processing has been most widely used as the primary method of food sterilization. In general, cooking at temperature above 60°C is required to inactivate *C. perfringens* vegetative cells in food (Doyle, 2002). To ensure elimination of spores in foods, thermal process at 100°C or higher are generally carried out. *C. perfringens* spores are more heat resistant than those of other strains and can survive even at 100°C for 1 h (Evelyn & Silva, 2016). However, heating at high temperature for long time may provide adverse effects on the quality of meat and meat products such as changes in color and texture, and nutritional loss.

Current strategies to control germination and outgrowth of *C. perfringens* involve chemical additives to overcome these unwanted side effects. Sodium nitrite has been used in meat products over the years to mainly control *Clostridium* species as well as flavor enhancer, color-fixing agent, and prevent lipid oxidation (Alahakoon

et al., 2015). However, World Health Organization (WHO) International Agency for Research on Cancer (IARC) announced that consumption of processed meat is probably carcinogenic to humans (WHO, 2015). Nitrite can cause the formation of carcinogenic *N*-nitrosoamines in the gastrointestinal tract (Butler, 2015). Therefore, increasing number of consumer is avoiding sodium nitrite due to the concerns of health risk of synthetic chemical additives (Yong et al., 2018). Currently, public attention has focused on the natural additives and novel processing technologies to control food-borne pathogens and increase shelf-life of meat products. For this reason, one of the main effort of the meat industry is to produce nitrite-free meat products because of a strong consumer demand. Although meat product without nitrite is microbiologically hazardous, nitrite-free meat products is on the market without safety guarantee. Therefore, a number of studies tried to find novel methods to inactivate *Clostridium* vegetative cells and spores.

2.1.3.2. Novel methods

Meat industry is developing alternative approaches to overcome traditional methods, including non-thermal sterilization technologies that meet consumer demand for ensured meat safety and enhanced food quality (Torres & Velazquez, 2008). Sterilization is defined as the process which can effect complete inactivation of microorganisms including spores (Li & Farid, 2016). Non-thermal sterilization technologies include HP, irradiation, pulsed electric field (PEF), ultraviolet (UV), ultrasound, and cold plasma. Irradiation and HP are being emerged non-thermal

technologies which processed products already in the market. Other technologies such as pulsed electric filed, ultraviolet, and cold plasma are still under development to commercialization (Misra, 2015).

Food irradiation is a process exposing food to a certain amount of ionizing radiations such as gamma-rays, electron beams, and X-rays from natural source (radioisotope) or machinery (linear accelerator) (Farkas et al., 2014). The dose of irradiation permitted by Korea Food and Drug Administration (KFDA) for commercial food applications is no more than 10 kGy, which is impossible to inactivate spores in food products. Moreover, in spite of the fact that food irradiation at low dose is not sufficient energy to induce radioactive, consumer misunderstands the irradiation technology as a non-safe food processing (Farkas et al., 2014). Therefore, HP is now often regarded as one of the major recent sterilization technology in food industry. However, because the non-thermal methods are mainly used in the large scale production to process bulk quantities of foods, the investment and operation costs of equipment is high when compared to thermal processing. Alternative methods also consider the use of natural additives in meat products such as vinegar (Valenzuela-Martinez et al., 2010).

2.2. High pressure

2.2.1. Use of high pressure in microbial control

High pressure (HP) is a non-thermal technology with less adverse effects on food compared with conventional thermal processing (Cullen et al., 2012). HP is achieved by pressurizing packaged food products, placed in a vessel and subjected to an extreme pressure, which is transmitted by fluid medium. Therefore, HP can process packaged foods which could prevent recontamination of final products. The pressure is transmitted evenly and instantaneously through the sample no matter which shape or size of food (Rendueles et al., 2011).

The microbial inactivation by HP has been extensively researched. The HP effectively inactivates the spoilage microorganism and important foodborne pathogens vegetative cells such as *Salmonella* spp, *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Staphylococcus aureus* (Table 1). This inactivation effect should lead to an increased shelf-life and improved safety of food products with minimal effect on quality properties. The basic principles of microorganism inactivation by HP are based on protein denaturation which results in enzyme inactivation (Bajovic et al., 2012). The HP induced cell membrane damage, protein denaturation and decrease of intracellular pH are responsible for the inactivation of microorganisms (Smelt, 1998). The cell membrane is constructed as a phospholipid bilayer and HP causes a phase transition, resulting the membrane is destabilized and the permeability is changing (Shimada et al., 1993).

Table 1. Previous studies on inactivation effect of foodborne pathogenic vegetative cells by high pressure (HP) combined with different treatments (adapted from Rendueles et al., 2011)

Strains	HP (MPa)	Time (min)	T[1)] (°C)	Reduction (Log_{10})	Source
Salmonella Enteritidis	345	10	35	8.22	Alpas et al. (2000)
Escherichia coli O157:H7	345	10	35	8.14	Alpas et al. (2000)
Listeria monocytogenes	500	1	400	3.8	Chen, (2007)
Staphylococcus aureus	600	15	20	3.0	Patterson et al. (1995)

[1)]T, Temperature

2.2.2. Application of high pressure in animal origin foods

In the meat industry, HP is used mainly as a non-thermal decontamination technology to improve the food safety of processed and cooked ready-to-eat (RTE) meat products (Evelyn et al., 2016). HP has become an industrial reality for the meat industry within the past twenty years (Bajovic et al., 2012) with high consumer acceptance in comparison to other non-thermal technology such as irradiation. Moreover, HP is used all over the world and processed product range is increasing in meat industry. A brief overview of the commercially available meat products using HP is given in Table 2.

Table 2. Examples of commercially available meat products using high pressure (HP) (adapted from Bajovic et al., 2012)

Product	**Manufacturer**	**Country**	**Source**
Fermented sausage	Cris Tim	Romania	Cris-Tim, (2012)
Sliced dry cured meat	Santa Maria Foods	Canada	Hiperbaric, (2012)
Oven roasted chicken	Ifantis	USA	Ikeuchi, (2011)
Deli meats	Creta Farm	Greece	Hiperbaric, (2012)

2.2.3. Limitation of high pressure

HP in the range of 400-600 MPa is typically applied to inactivate pathogenic and spoilage microorganisms vegetative cells and to extend food shelf-life in food industry. Pressure treatment usually performed at room or lower temperatures to maintain food quality. However, it is not possible to apply enough to control microbial spores, especially highly resistant strains such as *C. perfringens*. Bacterial spores can survive even an applied pressure of 1200 MPa at room temperature (Larson et al., 1918). Therefore, pressure combined with mild or high temperatures is required to inactivate bacterial endospores, referred to as pressure assisted thermal sterilization (PATS) or high pressure thermal processing (HPTP). From the previous studies, inactivation effect of PATS was confirmed (Table 3). It is assumed that,

pressure can induce spore germination under moderate conditions, and then germinated spores will be thermally inactivated (Li et al., 2016). However, pressure levels above 1000 MPa or high temperature is not yet used at an industrial scale and also cause quality change of food. In economic aspects of food industry, also it can raise the process times and costs. Since, HP could not provide all the functions that sodium nitrite alone could do, it can be suggested to use other technology in combination to control *C. perfringens*.

Table 3. Previous studies on inactivation effect of foodborne pathogenic spores by high pressure (HP) combined with different treatments (adapted from Rendueles et al., 2011)

Strains	**HP (MPa)**	**Time (min)**	**T[1)] (°C)**	**Reduction (Log_{10})**	**Source**
Bacillus cereus	400	25	30	0.5	McClemens et al. (2001)
C. perfringens	500	30	25, 45, and 65	Minimal or no reduction	Papafragkou et al. (2002)
C. perfringens type A	650	15	75	3.7	Paredes-Sabja et al. (2007)
C. botulinum type A	827	20	75	2-3	Reddy et al. (2003)

[1)]T, Temperature

2.3. Vinegar

2.3.1. Use of vinegar in microbial control

Organic acids are used as food preservatives and have been found to act as inhibitory agents for *C. perfringens* (Talukdar, 2017). Organic acids such as acetic acid is the most studied compound, due to the fact that it is strictly related to safety. Therefore, acetic acid which is mostly used as vinegar, has been successfully applied for microbial inhibition in variety of foods (Ray, & Bhunia, 2008).

Vinegar is natural antimicrobial additives in foods and classified as Generally Recognized as Safe (GRAS) substance. It have significant bactericidal effects against both gram-positive and gram-negative bacteria. Xi et al. (2012) reported that frankfurter sausages manufactured with vinegar, lime, and cherry significant reduce the *Listeria monocytogenes*. Furthermore, buffered vinegar alone were effectively inhibiting the germination and outgrowth of *C. perfringens* spore in ground turkey roast without sodium nitrite. Other study reported that a blend of vinegar and buffered lemon juice was effective in controlling of *C. perfringens* spores in reduced-NaCl roast beef during abusive cooling conditions, regardless of the NaCl added and the cooling time used (Valenzuela-Martinez et al., 2012). The vinegar is able to pass through the microorganisms cell membranes and lead to bacterial cell death (Budak et al., 2014).

2.3.2. Application and limitation of vinegar in animal origin foods

Organic acids are used as food preservatives and have been found increasing shelf-life. One of them, a variety of natural vinegar has been long used as acidic seasoning around the world (Johnston et al., 2006). The earliest known use of vinegar dates back more than 10000 years and vinegar has been produced as a commercial product for approximately 5000 years ago (Budak et al., 2014). Pickling is a traditional method of preserving foods in vinegar which prevent spoilage and enhance desirable characteristics such as taste and flavor. Meat products preserved with vinegar, such as pickled sausages and pig's feet are cooked meat soaked in brine containing vinegar (Zaika, 2002). Vinegar helps break down the tough fibers in the meat as well as flavoring and antimicrobial agents. Meat pickling provide ready-to-eat (RTE) meat product, highly acceptable convenience with good shelf-life stability at room temperature (Arun et al., 2007).

Vinegar contains 5 to 40% acetic acid and other compounds that give the aroma characteristic (Ray et al., 2008). However, high concentrations of vinegar addition can alter other sensorial properties due to strong flavor. Therefore, minimal quantities of vinegar addition is required to avoid adverse effect on meat products. Since, low concentrations of vinegar cannot effectively inhibit bacteria, using other combination treatment is required. Moreover, vinegar have been shown to contain antioxidant, anticancer compounds, bioactive metabolites as well as antimicrobial effect (Choi et al., 2015). Fukuyama et al. (2007) reported that kurozu (i.e., a traiditional Japanese black vinegar which are produced from rice) has effectively inhibit tumor growth,

lipid peroxidation, and inflammation.

Vinegar can be manufactured worldwide using different food materials such as unpolished rice and fruits. Therefore, meat products added with vinegar can be used in the manufacturing of clean-label (i.e., products that do not contain ingredients that can be classified as chemicals) and where the ingredients listed must be recognizable to consumer (Park et al., 2014). Consumers typically prefer natural additives in meat products and perceive these meat products as natural food.

Chapter III.

Combined effect of high pressure and vinegar addition on the control of *Clostridium perfringens* and quality in emulsion-type sausage

3.1. Introduction

For the past few years, *Clostridium* infection has been a major health hazard worldwide. *Clostridium* is an anaerobic spore-forming bacterium, toxins of which cause severe illness and even death (Grass et al., 2013). The Centers for Disease Control and Prevention (CDC) reported that approximately 965,958 cases of foodborne-illness related to *Clostridium perfringens* infection occur annually in the United States (Juneja et al., 2013). In other countries, including Australia and Japan, *C. perfringens* is also one of the main reasons of bacterial foodborne outbreaks (Gould et al., 2004; Monma et al., 2015).

Meat and meat products are the major food items implicated in *C. perfringens* outbreak. As *C. perfringens* requires more than 12 different amino acids and oxygen-free environment for growth, it thrives in vacuum-packaged meat products (Bauer et

al., 1981; Park et al., 2014). Fortunately, vegetative cells of *C. perfringens* can be inactivated during cooking processes at temperature over 75°C; however, its spores are extremely heat resistant and can survive at 100°C for 1 h or more (Labbé, 2000). Spores also have short generation time (i.e., 8 min at the optimal growth temperature of 43-45°C), and germinate easily when the products are improperly cooled or temperature abused after cooking. Therefore, methods for controlling *Clostridium* population in packaged meat products post-cooking are urgently required (Labbé, 2000; Juneja et al., 2013).

Currently, nitrite addition is the most effective method for controlling the growth of vegetative cells and spores of *Clostridium*. Thus, synthetic nitrite including sodium and potassium nitrite have been generally used in meat products (Honikel, 2008). However, owing to the growing concern over the use of synthetic food additives, the number of consumers avoiding synthetic nitrite-containing food products is also increasing (Lee et al., 2017). As meat products without nitrite are microbiologically hazardous, several studies have attempted to develop new methods of inactivating the vegetative cells and spores of *Clostridium* (Dutra et al., 2016; Valenzuela-Martinez et al., 2010).

High pressure (HP) treatment is a commercial non-thermal sterilization technology capable of inactivating bacterial vegetative cells. However, compared to vegetative cells, most microbial spores are extremely HP resistant and can withstand more than 1000 MPa pressure (Smelt, 1998). In particular, *Clostridium* spores tend to be more pressure resistant than other spores, including those of *Bacillus* (Considine et al., 2008). Thus, HP should be combined with other treatments to inactivate both

vegetative cells and spores of *C. perfringens*.

Traditionally, vinegar has been used in food processing to provide antimicrobial activity and functionality (such as flavor enhancement or antioxidant activity) (Choe & Kim, 2016; Valenzuela-Martinez et al., 2010). Recently, Smith et al. (2018) reported that buffered vinegar inhibited outgrowth of *C. perfringens* spores in roast beef. However, the addition of large amounts of vinegar can lead to undesirable changes in the sensorial qualities of the final meat product, such as color and flavor. We hypothesized that combination treatment with HP and appropriate amounts of vinegar can inhibit *Clostridium* growth in meat products without adverse effects on quality. Therefore, the objective of present study was to evaluate the combined effects of HP and vinegar treatment on inhibition of *C. perfringens* growth in nitrite-free cooked emulsion-type sausage under poor temperature control and to assess the changes in its physicochemical properties for 5 weeks of storage.

3.2. Materials and methods

3.2.1. Sample preparation and experimental design

3.2.1.1. Manufacture of emulsion-type sausage

Pork hind leg meat and back fat were purchased from a commercial butcher (Seoul, Korea) and ground through a 5 mm plate (M-12S, Hankook Fujee Industries Co., Ltd., Hwaseong, Korea). Vinegar (Verdad® Avanta™ F100, Corbin/Purac, Gorinchem, Netherlands) was purchased from a commercial market.

In the present study, emulsion-type sausage was manufactured with 0, 1, and 2% vinegar and 0.02% sodium nitrite as the positive control. The ground meat was mixed with back fat, iced water, and additives in a silent cutter (Talsa K30, DSL Food Machinery Ltd., Valencia, Spain) depending on the formula of four treatments. The formulation of cooked emulsion-type sausage is presented in Table 1, and the manufacturing process is presented in Fig. 1. The temperature of the mixture was maintained below 10°C during the manufacturing process and was monitored using a digital thermometer (TM-747DU, Tenmars Electronics Co., Ltd., Taipei, Taiwan). After emulsification, each meat batter was stuffed in collagen casing (25 mm diameter; NDX, Viscofan, Ceske Budejovice, Czech Republic) and vacuum-packaged in linear low density polyethylene bags (LLDPE; 25 × 35 cm; oxygen permeability of 22.5 mL/m^2/24 h atm at 60% relative humidity/25°C; water vapor permeability of 4.7 g/m^2/24 h at 100% relative humidity/25°C). The packaged sausages were cooked in a water bath at 80°C for 30 min until the internal temperature

of the sausage reached 75°C for pasteurization. This ensured the absence of interference from endogenous bacteria prior to inoculation with *C. perfringens*. Next, the samples were cooled in iced water.

Manufactured sausages were randomly divided into two groups. One group was inoculated with *C. perfringens* vegetative cells and spores to confirm the antimicrobial effect of HP, vinegar, or combined treatments. The other group was used to analyze the quality properties of the sausages by the same effects as described previously. Subsequently, HP was applied to both groups except for sausages containing sodium nitrite (positive control).

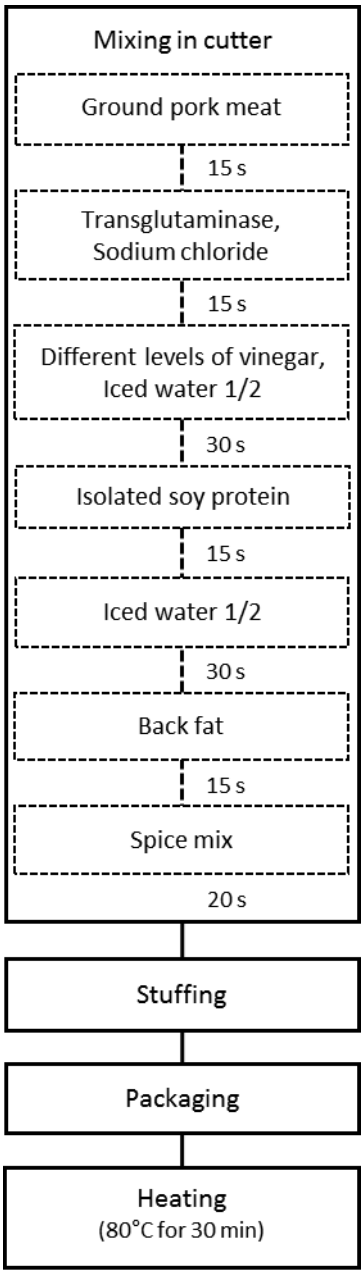

Fig. 1. Flow diagram for manufacturing process of emulsion-type sausages with different levels of vinegar

Table 1. Formulations (%) for manufacturing emulsion-type sausages with different levels of vinegar

Ingredients	Sodium nitrite (PC)[1)]	Addition level of vinegar		
		0%	1%	2%
Pork meat	66.34	66.34	66.34	66.34
Back fat	12.00	12.00	12.00	12.00
Iced water	17.70	17.70	17.70	17.70
Sodium chloride	1.20	1.20	1.20	1.20
Isolated soy protein	1.42	1.42	1.42	1.42
Spice mix	1.14	1.14	1.14	1.14
Transglutaminase	0.20	0.20	0.20	0.20
Total	100.00	100.00	100.00	100.00
Vinegar	-	-	1.00	2.00
Sodium nitrite	0.02	-	-	-

[1)]PC, Positive control

3.2.1.2. High pressure treatment and storage

Vacuum-packaged sausages were processed using the HP processing unit (VC-50, Innoway, Anyang, Korea), which was submerged in water as a hydrostatic fluid medium in the treatment chamber (cylinder Ø 200 mm in internal diameter, Ø 800 mm in external diameter, 2,250 mm in length, and working volume of 50 L). Then, the samples were pressurized with 4 cycles of 500 MPa each for 3 min. The time required for pressure to come up and release were 255 and 125 s, respectively. This equipment did not have temperature control inside the chamber, and hence only the initial and final temperature of sausage samples could be monitored using an infrared thermometer (ST-101, Sinyoung Choukki Co., Ltd., Bucheon, Korea). The temperatures of the sausages were maintained below 5°C. The non-pressurized samples were maintained under atmospheric pressure (0.1 MPa) and stored at 4°C. After HP treatment, sausages samples for microbial analysis were stored at 4°C for 2 weeks, following which the temperature was increased to 20°C and maintained at that temperature for 3 weeks. The sausages for quality analysis were stored at 4°C for 5 weeks. A diagram illustrating the experimental procedure is shown in Fig. 2.

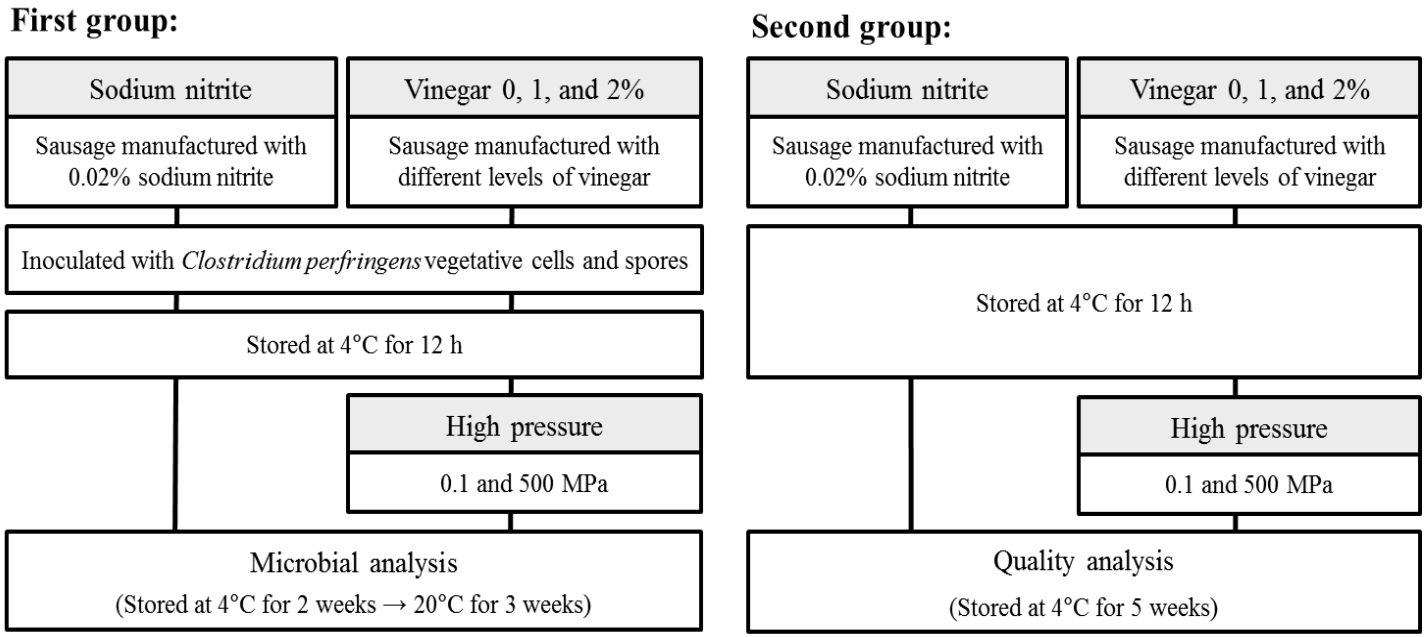

Fig. 2. Diagram illustrating the experimental procedure of the present study

3.2.2. Microbial analysis

3.2.2.1. Preparation of *C. perfringens*

Four *C. perfringens* type A strains (KCCM12098, KCCM40946, KCCM40947, and KCTC5101) were cultivated independently in cooked meat medium (CMM; Oxoid, Hampshire, UK) at 37°C for 24 h under anaerobic condition. One milliliter of each starter culture was transferred to 9 mL of brain heart infusion medium, and anaerobically incubated at 37°C for 24 h. Then, the four strains were mixed and centrifuged at 1,912 × *g* for 15 min at 4°C (Continent 512R, Hanil Co., Ltd., Incheon, Korea). The vegetative cells of the strains were washed twice and suspended in sterile phosphate buffer saline (PBS).

In order to obtain *C. perfringens* spores, four *C. perfringens* type A strains were also cultivated independently in CMM at 37°C for 24 h under anaerobic condition. The starter culture (100 μL) was transferred into 10 mL of fluid thioglycollate (FTG; Difco, Becton Dickinson, France) broth. The inoculated culture was heated at 75°C for 20 min to inactivate the vegetative cells, and incubated at 37°C for 18 h. Subsequently, the culture (100 μL) was transferred to freshly prepared FTG (10 mL) and incubated at 37°C for 4 h. The incubated culture (500 μL) was then transferred to 49.5 mL modified Duncan-Strong medium (4.5 g proteose peptone, 1.2 g yeast extract, 0.3 g sodium thioglycolate, 3 g sodium phosphate, 1.2 g raffinose, 15 mL caffine, and 300 mL distilled water) and anaerobically incubated at 37°C for 24 h. The culture of each strain

was centrifuged at 1,912 × *g* for 15 min at 4°C (Continent 512R) and washed twice with PBS. Finally, the spores were suspended in PBS. The spore crops were mixed in equal quantities before making a cocktail of strains for inoculation.

3.2.2.2. Inoculation

Before the HP treatment, sausage samples were inoculated with vegetative cells or spores of *C. perfringens*. Each sausage sample (10 g) was dipped in PBS (300 mL) containing the inoculum which was approximately 8 Log colony-forming units (CFU)/mL, and stirred for 2 min. After dipping, the samples were transferred to Petri dish and air dried for 10 min to allow attachment of *C. perfringens*. Then, the sausages were vacuum-packaged in LLDPE bags. After inoculation, the sausage samples were stored at 4°C for 12 h to give the spores an opportunity to germinate. The sausage samples were inoculated with an average of 4.4 ± 0.3 Log CFU/g for vegetative cells and 2.6 ± 0.3 Log CFU/g for spores.

3.2.2.3. Enumeration

After HP treatment, the number of vegetative cells and spores of *C. perfringens* in sausage samples were analyzed during the storage period of 5 weeks. Sausage sample (5 g) was added to 50 mL of 0.1% (w/v) sterile buffered peptone water (BPW; Becton, Dickinson and Company, Sparks, MD, USA) as

dilution fluid and the contents were homogenized for 2 min using a bag mixer (BagMixer; Interscience, St. Nom, France). Immediately after homogenization, the samples were serially ten-fold diluted with BPW. One hundred microliters of appropriate dilutions were spread plated in duplicate on tryptose-sulfite-cycloserine agar (TSC, Difco). After the plates were dried, they were overlaid with additional 10 mL of TSC and anaerobically incubated at 37°C for 18 h using the Gas Pak system (Oxoid Anaerogen 2.5 L Sachet, Thermo Fisher Scientific). Typical *C. perfringens* colonies were enumerated, and the counts were expressed as Log CFU/g of sausage sample.

3.2.3. Quality analysis

3.2.3.1. pH value

Each sausage sample (1 g) was homogenized with 9 mL of distilled water (DW) using a homogenizer (T10 basic, Ika Works, Staufen, Germany). The homogenates were filtered through filter paper (Whatman No. 4, Whatman International Ltd., Kent, UK) after centrifugation at 2,265 × *g* for 10 min at 4°C (Continent 512R). The pH value of filtrate was measured using a pH meter (SevenGo, Mettler-Toledo International Inc., Schwerzenbach, Switzerland).

3.2.3.2. Water holding capacity

The sausage sample (5 g) was placed on a filter paper (Whatman No. 4) in a centrifuge tube and centrifuged (Continent 512R) at 252 × *g* for 10 min. The

released water content was calculated as difference in sample weight before and after centrifugation and expressed as a percentage. Separately, the moisture content of sausages was determined according to Association of Official Analytical Chemists method (AOAC, 1995). Then, the water holding capacity (WHC) percentage was calculated using the following formula:

$$\text{Released water content (\%)} = \frac{\text{Weight before centrifuging - Weight after centrifuging}}{\text{Weight before centrifuging}} \times 100$$

$$\text{Water holding capacity (\%)} = \frac{\text{Moisture content - Released water content}}{\text{Moisture content}} \times 100$$

3.2.3.3. Instrumental color analysis

The surface color of sausages was measured using a colorimeter (CM-5, Konica Minolta Co., Ltd., Osaka, Japan) with a 3 mm measuring port. The instrument was calibrated using a standard black and white plate (CIE L^* = 96.74, CIE a^* = -0.11, CIE b^* = -0.15). Then, the color values were expressed as lightness (L^*), redness (a^*), and yellowness (b^*).

3.2.3.4. Texture profile analysis

Sausage samples (2.5 × 2.0 cm^2, diameter × height) were compressed twice to 60% of their original height using a TA1 texture analyzer (AMETEK Lloyd instruments Ltd., Fareham, UK) attached to a compression plate (70 mm in diameter) at a test speed of 2.0 mm/s and trigger force of 1 N. Texture analysis was performed using the NexygenPlus™ software (AMETEK Lloyd

instruments Ltd.), and the values of hardness (N), springiness, and chewiness (N) were recorded.

3.2.3.5. Measurement of 2-thiobarbituric acid reactive substances

Lipid oxidation was evaluated by calculating the concentration of malondialdehyde as a 2-thiobarbituric acid reactive substances (TBARS) value. The TBARS values of the sausage samples were measured according to method of Yong et al. (2017). Each sausage sample (5 g) was homogenized with 15 mL of DW and 50 μL of butylated hydroxytoluene (7.2% in ethanol) using a homogenizer (T10 basic). The supernatant (2 mL) was transferred to a test tube after centrifugation at 2,265 × g for 10 min and mixed with 4 mL of thiobarbituric acid (0.02 M)/trichloroacetic acid (15%) solution. Then, the test tubes were heated in a water bath at 90ºC for 30 min, cooled, and centrifuged (Continent 512R) at 2,265 × g for 15 min. The absorbance of the supernatant was measured at 532 nm using a spectrophotometer (X-ma 3100, Human Co. Ltd., Korea). The TBARS values were calculated using a standard curve of malondialdehyde (MDA) and expressed as mg MDA/kg sample.

3.2.4. Statistical analysis

All experiments were performed in triplicate with three batches. Statistical analysis was performed using one-way analysis of variance, and significant differences between mean values were identified using Student-Newman-Keuls

multiple range test of the SAS statistical software (SAS, Release 9.4; SAS Institute Inc., Cary, NC, USA) with a significance level of $P<0.05$. In addition, a multifactorial analysis of variance using the general linear model was applied to investigate the effect of vinegar concentration (0, 1, and 2 %) and HP (0.1 and 500 MPa).

3.3. Results and discussion

3.3.1. Microbial analysis

The HP and vinegar treatment in emulsion-type sausages differentially affected growth inhibition of the vegetative cells and spores of *C. perfringens* (Tables 2 and 3). HP treatment in emulsion-type sausages induced the significant reduction in number of vegetative cells of *C. perfringens*, while no significant reduction in its spores. The initial amount of inoculated vegetative cells ranged from 4.0 to 4.6 Log CFU/g (data not shown). Immediately after HP treatment, all treatments significantly reduced the vegetative cell number of *C. perfringens* in emulsion-type sausages. Regardless of vinegar addition, HP treatment showed lower counts of vegetative cells for 2 weeks at 4°C (P<0.05).

To assess whether the treatments applied could effectively control the growth of *C. perfringens*, samples stored for 2 weeks at 4°C and then moved to incubator from 3 to 5 weeks at 20°C. Two storage temperatures were selected to simulate proper refrigeration conditions (4°C) and temperature abuse (20°C), respectively, which represented the typical "poor control" conditions encountered during distribution and storage of the product in the food chain that possibly favor spore germination and outgrowth. Temperature fluctuations are frequently observed in commercial and home refrigerators, which can positively affect the survival, germination, and growth of *C. perfringens* contaminating vacuum-packaged products (Limbo et al., 2010). The group without HP and vinegar treatment showed rapid growth of vegetative cells,

reaching 7.2 Log CFU/g at the end of storage period, although HP treatment showed 3.8 Log CFU/g. Therefore, HP can be used to reduce the initial number and slow down the growth of *C. perfringens* vegetative cells compared to the non-pressurized group (0.1 MPa).

The addition of vinegar in emulsion-type sausages led to significant reduction in growth of *C. perfringens* vegetative cells and spores regardless of the addition levels (Tables 2 and 3). The vegetative cell population was maintained during the storage period in samples treated with vinegar alone and in combination with HP treatment, and showed no significant difference with the sodium nitrite group during temperature abusive storage (Table 2). At the end of the storage period, the vegetative cell counts did not vary significantly between groups with HP and/or vinegar treatment and those containing sodium nitrite ($P>0.05$).

The number of *C. perfringens* spores in emulsion-type sausages did not vary significantly among the treatments for 2 weeks at 4°C (Table 3). The addition of vinegar (1 and 2%, respectively) in sausages maintained the population of *C. perfringens* spores for 5 weeks, even after temperature abuse at 20°C, regardless of HP treatment. However, the sausages without vinegar showed rapid increase in the number of *C. perfringens* spores, which was higher than that in the sodium nitrite group, regardless of HP treatment ($P<0.05$). These results suggest that the addition of vinegar inhibits the germination and outgrowth of *C. perfringens* spores over the entire shelf life of 5 weeks. These findings are in agreement with the results of Valenzuela-Martinez et al. (2010),

who reported that vinegar is effective in controlling germination and outgrowth of *C. perfringens* spores in ground turkey roast without sodium nitrite. Similarly, Li et al. (2012) reported that the number of *C. perfringens* spores in sodium-roasted beef was controlled by vinegar treatment during abusive exponential cooling.

In our preliminary study, 0.5% vinegar was added to optimize its addition level in emulsion-type sausages, as mentioned (Table 1). There were no significant differences in inactivation of *C. perfringens* vegetative cells and spores by vinegar treatment during storage (0, 1, 2, 4, 6, and 9 days) at 4°C (data not shown). However, in the present study, vinegar treatment inhibited the growth of *C. perfringens* regardless of addition levels of vinegar (1 and 2%) during storage period. Therefore, 1% vinegar addition level may be sufficient for inactivating *C. perfringens* in emulsion-type sausages.

In overall, the HP treatment in emulsion-type sausages is efficient for the inactivation in vegetative cells but not in spores of *C. perfringens*. According to previous studies, the HP treatment at 400-600 MPa in less than 10 min is typically used for the inactivation of spoilage and pathogenic microorganisms in meat products and extension of shelf-life (Bajovic et al., 2012; Evelyn & Silva, 2016). Therefore, in this study, the sausage samples were pressurized with 4 cycles of 500 MPa each for 3 min. This experimental condition was based on the results of other studies showing that multiple-cycle HP treatment was more effective than single-cycle treatments for inactivating pathogenic microorganisms in raw meat (Morales et al., 2008; Morales et al., 2009).

However, spores are highly resistant to pressure, and are capable of surviving in up to 1200 MPa pressure (Larson et al., 1918). Furthermore, in this study, the HP treatment in sausage samples without vinegar addition showed earlier spore germination after 3 to 5 weeks at 20°C (Table 3). This can be partially explained by the results of previous studies showing that HP treatment can lead to germination of spores of different microorganisms. This is probably caused by pressurization between 500-600 MPa induce the spore germination by opens channels for the release of dipicolinic acid (DPA) from the spore core (Paidhungat et al., 2002; Setlow, 2003). In addition, application of two cycles of pressure at 500 MPa slightly increased *Bacillus* spore germination compared to the use of single pressure cycle (Black et al., 2008). Therefore, treatment with a combination of HP and vinegar, but not HP alone, was required for inactivating *C. perfringens* spores. Sijtsema et al. (2017) found that treatment with a combination of vinegar and jasmine tea extract controlled the growth of total aerobic bacteria and *Salmonella* and provided a clean label option for the meat industry (i.e., products that do not contain chemical ingredients). In this study, the addition of vinegar significantly inactivated the vegetative cells and spores of *C. perfringens* in emulsion-type sausages, showing similar values to that of the sodium nitrite-treated group regardless of HP treatment. Therefore, it can fit the definition of clean label, while providing the antimicrobial effect and assure its safety.

Table 2. Effect of high pressure (HP) and vinegar addition on the *Clostridium perfringens* vegetative cell counts (Log CFU/g) in emulsion-type sausage

HP (MPa)	Treatments	Storage at 4°C (weeks)			Storage at 20°C (weeks)			SEM[1)]
		0	1	2	3	4	5	
0.1	Sodium nitrite	4.1^{Ba}	3.9^{Aa}	4.1^{Aa}	2.8^{Bb}	2.5^{Cb}	3.2^{BCab}	0.293
	0% vinegar	4.6^{Ac}	3.6^{ABd}	4.0^{Acd}	5.5^{Ab}	7.2^{Aa}	7.2^{Aa}	0.200
	1% vinegar	3.8^{BCa}	3.6^{ABa}	3.2^{Bb}	2.7^{Bc}	2.5^{Cc}	2.7^{BCc}	0.112
	2% vinegar	3.7^{Ca}	3.3^{Bab}	3.1^{Bb}	2.6^{Bc}	2.6^{Cc}	2.3^{Cc}	0.119
500	0% vinegar	2.7^{Dab}	2.6^{Cab}	2.3^{Cb}	4.0^{ABab}	4.4^{Ba}	3.8^{Bab}	0.412
	1% vinegar	2.5^{D}	2.4^{C}	2.4^{C}	2.5^{B}	2.3^{C}	2.6^{BC}	0.118
	2% vinegar	2.6^{D}	2.5^{C}	2.3^{C}	3.2^{B}	2.5^{C}	2.4^{C}	0.355
SEM[2)]		0.118	0.131	0.113	0.484	0.166	0.300	

[1)]Standard error of the mean (n=18), [2)](n=21).

[A-D]Values with different letters within the same column differ significantly (P<0.05).

[a-d]Values with different letters within the same row differ significantly (P<0.05).

Table 3. Effect of high pressure (HP) and vinegar addition on the *Clostridium perfringens* spore counts (Log CFU/g) in emulsion-type sausage

HP (MPa)	Treatments	Storage at 4°C (weeks)			Storage at 20°C (weeks)			SEM[1)]
		0	1	2	3	4	5	
0.1	Sodium nitrite	2.6	2.7	2.5	2.9^{CD}	3.9^{B}	2.5^{C}	0.387
	0% vinegar	2.6^{b}	2.1^{b}	2.6^{b}	5.4^{Ba}	5.2^{ABa}	5.1^{Ba}	0.506
	1% vinegar	2.7	2.7	2.7	2.6^{D}	2.4^{B}	2.8^{C}	0.116
	2% vinegar	2.5	2.7	2.6	2.3^{D}	2.2^{B}	2.3^{C}	0.213
500	0% vinegar	2.6^{b}	2.6^{b}	2.2^{b}	6.2^{Aa}	7.0^{Aa}	6.7^{Aa}	0.231
	1% vinegar	2.3	2.4	2.5	3.6^{C}	4.3^{B}	2.5^{C}	0.627
	2% vinegar	2.2	2.5	2.5	2.2^{D}	2.6^{B}	2.3^{C}	0.178
SEM[2)]		0.135	0.296	0.155	0.241	0.648	0.448	

[1)]Standard error of the mean (n=18), [2)](n=21).

[A-D]Values with different letters within the same column differ significantly (P<0.05).

[a,b]Values with different letters within the same row differ significantly (P<0.05).

3.3.2. Quality analysis

3.3.2.1. pH and WHC

The addition of vinegar significantly increased pH values in emulsion-type sausages as increasing vinegar concentration regardless of HP treatment and storage period (Table 4). The sausages added with 2% vinegar induced the highest pH values among the treatments and it might be due to inherently high pH of vinegar (7.50). The pH values were dependent on the addition of vinegar and its addition level; however, changes in pH values were not meaningful because of small extents even though there was significant. Similarly, Sijtsema et al. (2017) reported that addition of vinegar containing jasmine tea extract in chicken patties slightly increased the pH values.

The WHC indicating water-retaining ability is one of the important factors determining meat quality, and affects its sensory properties (Van Oeckel et al., 1999). HP and/or vinegar treatment resulted in significantly higher WHC during 5 weeks of storage compared to sodium nitrite addition group (Table 4). The highest WHC (P<0.05) was observed in sausage samples with HP and vinegar treatment regardless of the addition level of vinegar. This result might be partially affected by the increase in pH values originated from the vinegar addition (P=0.0092, Table 8). Also, HP treatment in sausage samples significantly influenced on the increase in WHC (P=0.0009, Table 8). A previous study reported that HP treatment enhanced WHC of restructured pork meat (Hong et al., 2006). In addition, Grossi et al. (2012) found that HP

treatment of meat products increased myofibrillar protein solubility by disrupting electrostatic and hydrophobic interactions and hydrogen bonding, which increased WHC. In general, initial WHC of all treatment was maintained until 1 week, which peaked at week 3, and decreased thereafter until end of storage. However, there were no significance differences on cooking loss and processing yield among the treatments (data not shown, $P>0.05$).

Table 4. Effect of high pressure (HP) and vinegar addition on the pH and water holding capacity (WHC) in emulsion-type sausage stored at 4°C

Traits	HP (MPa)	Treatments	Storage (weeks)				SEM[1)]
			0	1	3	5	
pH	0.1	Sodium nitrite	6.25^{Cb}	6.24^{Db}	6.26^{Dab}	6.28^{Da}	0.006
		0% vinegar	6.27^{Ba}	6.20^{Ec}	6.26^{Da}	6.24^{Eb}	0.004
		1% vinegar	6.28^{Bb}	6.26^{Cc}	6.31^{Ba}	6.31^{Ca}	0.003
		2% vinegar	6.35^{Ab}	6.33^{Ac}	6.34^{Ac}	6.36^{Aa}	0.003
	500	0% vinegar	6.24^{C}	6.24^{D}	6.23^{E}	6.24^{E}	0.002
		1% vinegar	6.29^{B}	6.28^{B}	6.29^{C}	6.29^{D}	0.004
		2% vinegar	6.35^{Aa}	6.33^{Ac}	6.34^{Ab}	6.34^{Bb}	0.002
	SEM[2)]		0.004	0.003	0.005	0.004	
WHC (%)	0.1	Sodium nitrite	74.50^{Eb}	73.28^{Cb}	87.71^{Da}	72.49^{Cb}	2.553
		0% vinegar	78.16^{Db}	77.77^{Bb}	89.31^{Ca}	74.10^{Cb}	1.126
		1% vinegar	80.84^{Bb}	77.26^{Bc}	89.82^{BCa}	79.37^{BCb}	0.557
		2% vinegar	80.53^{Bb}	78.90^{Bb}	91.53^{Aa}	78.85^{BCb}	0.475
	500	0% vinegar	79.75^{Cc}	78.02^{Bc}	89.23^{Ca}	82.21^{ABb}	0.629
		1% vinegar	84.20^{Ac}	83.00^{Ad}	90.92^{ABa}	87.68^{Ab}	0.232
		2% vinegar	84.74^{Ab}	83.00^{Ab}	91.78^{Aa}	83.85^{ABb}	0.495
	SEM[2)]		0.193	1.066	0.368	1.961	

[1)]Standard error of the mean (n=12), [2)](n=21).

[A-E]Values with different letters within the same column differ significantly ($P<0.05$).

[a-d]Values with different letters within the same row differ significantly ($P<0.05$).

3.3.2.2. Texture profile analysis

Texture characteristics are important in the processed meat products because they affect consumer acceptability (Chan et al., 2011). In this study, the texture properties (hardness, springiness, and chewiness) were partially affected by HP and/or vinegar treatment (Table 5). Regardless of HP treatment, the sausage samples without vinegar generally showed similar hardness and chewiness (P>0.05) to sodium nitrite-treated samples during the entire storage period, except for hardness in 3 and 5 weeks.

Vinegar treatment in sausage samples induced significant increase in hardness and it might be due to the greater emulsifying as forming a three-dimensional network by the vinegar addition. The emulsion stability of mayonnaise dressing increased with increase in vinegar concentration (Yang, and Han, 2002). Kumar and Tanwar (2011) reported that chicken nuggets added with mustard showed higher sensory texture scores due to higher emulsification and WHC of mustard. Similar values (P>0.05) in springiness were observed among the treatments at week 5 except for sausage samples without HP and vinegar treatment. Vinegar treatment in sausage samples led to decrease and increase in chewiness at the initial and later storage time points, respectively. In general, HP treatment in sausage samples resulted in significantly higher chewiness among the treatments relatively. Fernandez et al. (1998) reported that pressurization of chicken meat batters at 200 MPa and 70°C increased hardness and chewiness compared to non-pressurized samples. A previous study reported that cooked sausages pressurized at 500 MPa were less firm than untreated ones

(Mor-Mur & Yueste, 2003). In this study, the HP treatment did not critically affect changes in texture, and the cooking process possibly induced gelation before HP treatment. This may minimize the effect of HP treatment on changes in texture properties (Mor-Mur & Yueste, 2003).

Table 5. Effect of high pressure (HP) and vinegar addition on the texture analysis in emulsion-type sausage stored at 4°C

Traits	HP (MPa)	Treatments	Storage (weeks)				SEM[1)]
			0	1	3	5	
Hardness (N)	0.1	Sodium nitrite	46.73^{CDb}	58.70^{Ca}	47.75^{Eb}	47.32^{Cb}	2.332
		0% vinegar	43.48^{Dc}	60.03^{BCa}	49.97^{DEb}	47.00^{Cbc}	1.107
		1% vinegar	63.62^{Bb}	71.30^{BCb}	81.31^{Ba}	69.14^{Ab}	2.998
		2% vinegar	70.94^{A}	75.57^{B}	70.26^{C}	73.51^{A}	1.788
	500	0% vinegar	51.37^{C}	64.40^{BC}	56.80^{D}	54.35^{B}	4.204
		1% vinegar	63.64^{Bd}	91.33^{Aa}	79.81^{Bb}	73.25^{Ac}	1.168
		2% vinegar	64.57^{Bc}	68.96^{BCbc}	92.23^{Aa}	74.76^{Ab}	2.421
	SEM[2)]		1.712	3.608	2.308	1.928	
Springiness	0.1	Sodium nitrite	0.89^{Aa}	0.84^{Aa}	0.75^{Aa}	0.43^{Bb}	0.073
		0% vinegar	0.72^{Ba}	0.84^{Aa}	0.68^{Aa}	0.52^{Ab}	0.046
		1% vinegar	0.60^{BC}	0.60^{AB}	0.40^{C}	0.39^{B}	0.056
		2% vinegar	0.60^{BCa}	0.59^{ABa}	0.49^{BCb}	0.41^{Bc}	0.022
	500	0% vinegar	0.68^{BCa}	0.59^{ABab}	0.61^{ABab}	0.40^{Bb}	0.057
		1% vinegar	0.57^{BCa}	0.45^{Bb}	0.46^{BCb}	0.35^{Bc}	0.016
		2% vinegar	0.52^{Ca}	0.54^{ABa}	0.50^{BCa}	0.36^{Bb}	0.016
	SEM[2)]		0.039	0.068	0.044	0.019	

(Continued on next page)

Table 5. (Continued)

Traits	HP (MPa)	Treatments	Storage (weeks)				SEM[1)]
			0	1	3	5	
Chewiness (N)	0.1	Sodium nitrite	8.05^{AB}	11.78^{B}	7.35^{C}	7.68^{D}	1.192
		0% vinegar	6.80^{B}	9.87^{B}	8.54^{C}	8.99^{CD}	0.818
		1% vinegar	9.04^{ABb}	11.43^{Bab}	15.42^{Ba}	16.23^{ABa}	1.287
		2% vinegar	10.78^{Ab}	12.01^{Bb}	8.12^{Cb}	18.97^{Aa}	1.446
	500	0% vinegar	9.85^{AB}	9.92^{B}	8.66^{C}	12.67^{BC}	1.884
		1% vinegar	9.20^{ABb}	16.40^{Aa}	11.80^{BCb}	17.99^{Aa}	0.915
		2% vinegar	9.20^{ABb}	8.10^{Bb}	20.53^{Aa}	20.18^{Aa}	0.535
	SEM[2)]		0.886	1.204	1.438	1.306	

[1)]Standard error of the mean (n=12), [2)](n=21).

[A-D]Values with different letters within the same column differ significantly (P<0.05).

[a-d]Values with different letters within the same row differ significantly (P<0.05).

3.3.2.3. Instrumental color analysis

The color of meat products is an important factor attributing consumers' purchase decisions (Mancini & Ramanathan, 2008). Changes in color (L^*, a^*, and b^*) were dependent on HP, vinegar, and their combination treatment during storage of 5 weeks (Table 6). The HP and vinegar treatment, independently, in sausage samples decreased L^* values during the entire storage period except for samples treated with HP at initial storage time. This indicates that cooked emulsion-type sausages tended to be darker. Similarly, Fernandez et al. (1998) reported decrease in L^*, a^*, and b^* values was observed in chicken batters by HP treatment. This discoloration has been associated with pressure, which induces modifications in hemoprotein, sarcoplasmic, and myofibrillar proteins of meat (Cheftel & Culioli, 1997). Furthermore, the reduction in L^* values was possibly due to the brownish tint color of the vinegar which was used in this study. This finding was different from that of Sikes et al. (2009) who found an increase in L^* value of pressurized beef sausage batters. However, when the product was cooked, myoglobin was converted into a nitrosyl-hemochromogen pigment, which is not affected by pressure. (Carlez et al., 1995; Mor-Mur & Yueste, 2003). This is the reason why color parameter of L^* almost did not increase in this work.

As expected, sausage samples with sodium nitrite had the highest a^* values (P<0.05) among the treatments during the entire storage period. The a^* values were not generally affected (P>0.05) by HP treatment during storage, but were affected (P<0.05) by vinegar treatment at 3 and 5 weeks. In contrast, Yuste et al. (1999)

reported that cooked sausages treated with HP at 500 MPa had lower a^* and b^* values compared to non-treated one. In general, the highest and lowest values (both $P<0.05$) of a^* were observed for sausage samples treated with sodium nitrite and vinegar, respectively. The vinegar treatment only affected to a decrease in b^* values and it indicates that the color of the product turned blue rather than yellow.

In overall, addition of vinegar changed the color attributes of the emulsion-type sausages by tarnishing the sample. These changes in L^*, a^*, and b^* values by vinegar treatment were probably due to its inherent color (L^*; 25.60, a^*; 6.12, b^*; 20.71). The color of the final products is mostly influenced by the color of the added ingredients. Color stability is critical for sausages because consumers use discoloration as an indicator of spoilage (Mancini & Ramanathan, 2008). In general, the difference between initial and final storage times was not significant for the color of sausages in this study. The use of natural ingredients instead of synthetic additives, which can be noticed on the label of such products, may be more attractive for consumers (Bruhn, 2007).

Table 6. Effect of high pressure (HP) and vinegar addition on the color analysis in emulsion-type sausage stored at 4°C

Traits	HP (MPa)	Treatments	Storage (weeks)				SEM[1)]
			0	1	3	5	
L^*	0.1	Sodium nitrite	67.49^{Aa}	66.80^{Aa}	67.10^{Ba}	64.83^{Bb}	0.234
		0% vinegar	67.39^{Aab}	66.71^{Ab}	68.51^{Aa}	67.06^{Aab}	0.436
		1% vinegar	64.68^{B}	64.16^{C}	65.38^{C}	64.71^{B}	0.389
		2% vinegar	64.83^{B}	63.75^{C}	63.97^{D}	63.70^{B}	0.364
	500	0% vinegar	67.38^{Aa}	65.67^{Bb}	67.35^{Ba}	64.70^{Bb}	0.394
		1% vinegar	66.23^{ABa}	63.76^{Cb}	64.86^{CDb}	62.34^{Cc}	0.400
		2% vinegar	64.83^{Ba}	63.01^{Cb}	62.93^{Eb}	62.05^{Cb}	0.348
		SEM[2)]	0.438	0.306	0.338	0.390	
a^*	0.1	Sodium nitrite	8.13^{Ac}	8.33^{Abc}	8.57^{Aab}	8.80^{Aa}	0.124
		0% vinegar	3.45^{BCb}	3.89^{Ca}	3.97^{Ba}	4.11^{Ba}	0.130
		1% vinegar	3.06^{C}	3.20^{C}	3.17^{C}	3.01^{D}	0.118
		2% vinegar	3.16^{C}	3.29^{C}	3.49^{C}	3.45^{C}	0.148
	500	0% vinegar	3.74^{B}	4.63^{B}	4.11^{B}	4.41^{B}	0.300
		1% vinegar	2.91^{Cb}	3.23^{Cab}	3.49^{Ca}	2.78^{Db}	0.136
		2% vinegar	3.39^{BC}	3.17^{C}	3.28^{C}	3.42^{C}	0.120
		SEM[2)]	0.140	0.244	0.122	0.122	

(Continued on next page)

Table 6. (Continued)

Traits	HP (MPa)	Treatments	Storage (weeks)				SEM[1)]
			0	1	3	5	
b^*	0.1	Sodium nitrite	12.02^{Cc}	11.83^{Cc}	13.30^{Ca}	13.93^{Cb}	0.134
		0% vinegar	14.77^{Ab}	14.65^{Ab}	16.16^{Aa}	15.18^{Bb}	0.250
		1% vinegar	13.05^{Bb}	13.27^{Bb}	14.37^{BCa}	13.67^{Cab}	0.261
		2% vinegar	13.01^{Bb}	13.03^{Bb}	14.09^{BCa}	13.52^{Cb}	0.156
	500	0% vinegar	15.37^{A}	14.87^{A}	15.61^{A}	15.79^{A}	0.242
		1% vinegar	13.32^{Bb}	13.50^{Bb}	14.71^{Ba}	13.57^{Cb}	0.221
		2% vinegar	13.67^{Bab}	13.07^{Bb}	13.74^{BCab}	14.01^{Ca}	0.236
		SEM[2)]	0.226	0.160	0.309	0.127	

[1)]Standard error of the mean (n=12), [2)](n=21).

[A-D]Values with different letters within the same column differ significantly (P<0.05).

[a-d]Values with different letters within the same row differ significantly (P<0.05).

3.3.2.4. Lipid oxidation

Lipid oxidation is one of the primary causes of quality deterioration in meat products during storage (Morrissey et al., 1998). In particular, meat and meat products have high content of unsaturated fatty acids that are susceptible to lipid degradation than other food material (Medina-Meza et al., 2014).

Generally, HP treatment induces lipid oxidation in food (Campus et al., 2008). In this study, the HP treatment in sausage samples accelerated the lipid oxidation (by approximately 3-fold) compared to other treatment (Table 7). Similarly, Cava et al. (2009) reported that HP treatment in meat products significantly increased TBARS values even at lower pressure (200 MPa). However, in this study, addition of vinegar decreased the lipid oxidation of sausage samples treated with HP. In addition, vinegar treatment in sausage samples maintained (P>0.05) the TBARS values, showing 0.36-0.43 mg MDA/kg samples during the entire storage period, which was similar to the value obtained for the sodium nitrite group. This is probably caused by the functional materials of vinegar with antioxidant activity, such as polyphenols and flavonoids, which is similar to that of sodium nitrite-containing meat products (Choe & Kim, 2016; Sakanaka & Ishihara, 2008). In addition, jasmine tea extract in vinegar contains catechins, which show strong antioxidant power (Benzie & Szeto, 1999; Kim et al., 2005). However, the addition of different levels of vinegar did not inhibit TBARS values during the entire storage period (P>0.05). In terms of inhibition of lipid oxidation, these results suggest that sausages added with 1% vinegar are enough to alternate sodium nitrite in emulsion-type sausage.

Table 7. Effect of high pressure (HP) and vinegar addition on the 2-thiobarbituric acid reactive substances (TBARS) values (mg malondialdehyde/kg sausage) in emulsion-type sausage stored at 4°C

HP (MPa)	Treatments	Storage (weeks)				SEM[1)]
		0	1	3	5	
0.1	Sodium nitrite	0.33^{B}	0.33^{D}	0.37^{C}	0.39^{C}	0.015
	0% vinegar	0.50^{Bc}	1.04^{Bb}	1.15^{Bb}	1.47^{Ba}	0.076
	1% vinegar	0.36^{B}	0.38^{C}	0.42^{C}	0.43^{C}	0.019
	2% vinegar	0.37^{B}	0.36^{CD}	0.39^{C}	0.39^{C}	0.011
500	0% vinegar	0.93^{Ad}	1.19^{ABc}	1.33^{Ab}	1.53^{Aa}	0.029
	1% vinegar	0.39^{Bb}	0.39^{Cb}	0.41^{Cab}	0.43^{Ca}	0.009
	2% vinegar	0.36^{Bb}	0.36^{CDb}	0.39^{Ca}	0.40^{Ca}	0.006
SEM[2)]		0.057	0.012	0.025	0.018	

[1)]Standard error of the mean (n=12), [2)](n=21).

[A-D]Values with different letters within the same column differ significantly (P<0.05).

[a-d]Values with different letters within the same row differ significantly (P<0.05).

Table 8. *P*-value comparison of the effect of high pressure (HP), vinegar addition, and storage weeks in the emulsion-type sausage stored at 4°C

Traits	*P*-value					
	HP	Vinegar	Storage	HP x Vinegar	HP x Storage	Vinegar x Storage
Clostridium perfringens						
Vegetative cell	0.0002	<0.0001	<0.0001	0.1773	0.0483	0.0080
Spore	0.0017	<0.0001	<0.0001	0.0023	0.0002	<0.0001
pH	0.0059	<0.0001	<0.0001	0.6978	0.0003	0.1365
WHC (%)[1)]	0.0009	0.0092	<0.0001	0.1667	0.2539	0.7289
Color						
L^*	<0.0001	<0.0001	<0.0001	0.5663	<0.0001	0.0440
a^*	0.0029	<0.0001	0.7681	0.0001	0.9726	0.9402
b^*	<0.0001	<0.0001	<0.0001	0.0002	0.3328	0.3596
Texture						
Hardness (N)	0.0003	<0.0001	<0.0001	0.4088	0.1198	0.0015
Springiness	0.0001	<0.0001	<0.0001	0.0363	0.0873	0.0008
Chewiness (N)	0.0354	<0.0001	<0.0001	0.8216	0.2471	
TBARS[2)]	0.0009	<0.0001	0.0457	<0.0001	0.9908	0.0812

[1)]WHC, water holding capacity; [2)]TBARS, 2-thiobarbituric acid reactive substances.

3.4. Conclusion

Present study showed that each HP and vinegar treatment in nitrite-free sausage inhibited the growth of vegetative cells and both vegetative cells and spores of *C. perfringens*, respectively, during the entire storage period. In addition, the vinegar treatment in sausage showed no significant difference with sodium nitrite in the populations of vegetative cells and spores of *C. perfringens*, even under abusive temperature storage at 20°C. Therefore, it is possible to replace sodium nitrite using vinegar, and in combination with HP treatment, in order to inhibit the *C. perfringens* in emulsion-type sausage. In conclusion, a combination of HP and vinegar treatment would extend the shelf-life and provide acceptable product quality in emulsion-type sausage, even though there is tarnished color. Hence, this method can be used for manufacturing safe products with clean label, which can be perceived as natural meat products. Further studies are required to assess the effect of these changes on antioxidant and sensory characteristics of emulsion-type sausage.

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Summary in Korean

초고압과 식초 병용처리가 소시지에 접종된 *Clostridium perfringens* 제어와 품질에 미치는 영향

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최근 소비자는 식육가공식품을 구매할 시 고려하는 사항으로 맛, 영양 그리고 가격보다도 안전성을 최우선으로 중시한다. 하지만 동물성 식품에 의한 식중독은 전세계적으로 매년 꾸준히 발병되고있다. 클로스트리디움 퍼프린젠스는 그람 양성, 혐기성 포자 형성균으로 주변 환경 및 사람과 동물의 장내에 존재하며 장에서 독소를 생성해 식중독을 일으킨다. 주요 원인 식품은 동물성 식품으로 특히 산소 함량이 낮은 진공 포장된 소시지, 햄 등이다. 클로스트리디움 퍼프린젠스는 미국에서 매년 약 백만 건 이상의 식중독을 유발하는 가장 흔한 식중독균으로 알려져 있으며 대표적 증상으로는 복통과 설사다.

이러한 클로스트리디움 퍼프린젠스를 제어하는 방법으로는 가열처리가 있지만 오랜 시간 100도 이상의 높은 온도에서 처리해야 하므로 식품의 외관 변형, 영양소 파괴 등의 품질 저하를 가져올 수 있으며 내열성 포자가 있어 가열한 후 장시간 실온에 방치하면 다

시 포자가 성장하여 식중독을 일으킬 수 있다.

따라서 오래전부터 식육가공업계에서 육색의 발색과 안정화, 풍미 향상, 산패취 발생 감소와 함께 주요 역할로써 식중독의 원인균인 클로스트리디움의 성장과 독소생성을 억제하는 아질산나트륨을 식육가공식품에 첨가해왔다. 그러나 최근 소비자들은 건강 지향적 욕구 증대와 식품안전에 대한 의식 고조로 화학적 합성 첨가물을 기피하고 있다. 이러한 소비자의 요구를 충족시키기 위하여 식육가공업계들은 아질산나트륨 무첨가 식육가공식품을 서로 앞세워 출시하고 있지만 클로스트리디움 등 안전성에대한 충분한 확신 없이 판매하고 있는 실정이다. 따라서 아질산나트륨의 기능을 효과적으로 대체할 수 있는 천연 물질 등 새로운 방법을 찾는 노력이 전세계적으로 계속되고 있다.

비가열 처리 기술 중 하나인 초고압 기술은 영양소 파괴를 최소화하면서 미생물을 효과적으로 제어할 수 있어 다양한 식품의 안전성 및 저장성을 높일 수 있는 가공 기술로 평가 받고 있다. 하지만 영양세포뿐만 아니라 포자를 제어하기 위하여는 1200 MPa 이상의 고압에서 처리하거나 열처리를 병용하여 장시간 처리해야 한다. 하지만 이는 식품 품질에 영향을 미치며 비용 처리 시간 등 경제적 손실 또한 따른다. 따라서 오늘날 식품 업계에서 주로 사용되는 500-600 MPa 압력만으로는 포자를 효과적으로 제어할 수 없기 때문에 다른 병용처리가 필요하다. 식초는 음식 고유의 맛과 풍미를 살려주며 영양 성분과 항산화 물질이 풍부, 항균 효과 또한 있다. 식초는 약 5000년전부터 식품에 첨가되어온 조미료로써 소비자의 친숙도가 높다. 따라서, 식초를 식품에 첨가할 시 소비자들은 크게 거부감 없이 천연 첨가물로 자연스럽게 인식한다.

따라서 본 연구에서는 초고압과 식초 병용처리가 유화형 소시지에 접종된 클로스트리디움 퍼프린젠스 제어 효과 및 품질 특성에

미치는 영향을 평가하였다. 부적절한 온도 관리 속 클로스트리디움 퍼프린젠스 성장 및 발아 억제 효과를 살펴보고자 병합처리 후 소시지 샘플을 4℃에서 2주동안 저장한 후 온도를 높여 20℃에서 3주에서 5주동안 저장하여 미생물 분석을 진행하였다. 품질 특성은 4℃에서 5주동안 저장한 후 평가하였다.

그 결과, 초고압 처리는 유화형 소시지의 클로스트리디움 퍼프린젠스 초기 영양세포 감균에 효과적이었다. 또한 식초 첨가 처리구는 20℃의 부적절한 온도 설정 하에서도 영양세포와 포자의 수가 아질산나트륨 대조구와 유의적 차이가 나지 않는 것을 보아 식초는 클로스트리디움 퍼프린젠스 포자 발아 억제에 효과적이었음을 확인할 수 있었다. 품질 분석 결과 식초 첨가 시 유화형 소시지의 pH와 보수력이 높아졌으며 지방산패 또한 억제하여 저장기간 동안 아질산나트륨 대조구와 유의적 차이를 나타내지 않았다. 하지만 색도는 식초 고유의 갈색빛 때문에 명도, 적색도 그리고 황색도가 감소하여 다소 갈변하는 경향을 보이며 조직감의경우 초고압에 의한 영향은 없었으나 식초 첨가에의해 경도가 높아지는 경향을 보였다.

결론적으로, 본 연구를 통하여 합성 아질산나트륨의 대안으로 초고압과 식초 병용처리가 유화형 소시지에 접종된 클로스트리디움 퍼프린젠스를 효과적으로 제어할 수 있으며 지방산패 또한 억제하여 저장성을 높일 수 있는 것으로 사료된다.

주요어: 식초, 아질산나트륨, 유화형 소시지, 초고압, 클로스트리디움 퍼프린젠스